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Evaluation of the effects of repeated fecal collection by manual stripping on the plasma cortisol levels, *TNF-α* gene expression, and digestibility and availability of nutrients from hydrolyzed poultry and egg meal by rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract

The determination of apparent nutrient digestibility and availability coefficients (ADC and AAC) of ingredients is an important process in the formulation of cost efficient diets for fish. Collection of feces by repeated manual stripping is commonly practiced. However, due to the stress and possible physical damage imposed on fish during repeated stripping, this practice may influence ADC and AAC values. Our study evaluates the effects of repeated stripping three times over a period of five days on the ADCs and AACs of nutrients from hydrolyzed whole poultry egg meal for rainbow trout. We also evaluated the effects of repeated stripping on the stress status (plasma cortisol), innate immune response (*tumor necrosis factor-α* (*TNF-α*) gene expression) and physical damage of the distal section of the digestive tract (histological examination) of rainbow trout (mean weight 308 g). Plasma cortisol levels were significantly elevated at the completion of each stripping event, indicating the procedure was stressful to rainbow trout. While the gene expression of *TNF-α* indicated a compounding chronic effect of the repeated stripping procedure on the innate immune response of rainbow trout. However, microscopic histological examination of the distal section of the digestive tract indicated no apparent sign of physical damage due to the repeated fecal stripping procedure. Dietary ADCs and AACs for dry matter, organic matter, protein, amino acids and energy were unaffected by repeated stripping. While ingredient ADCs and AACs for dry matter, organic matter, protein, amino acids and lipid were also unaffected by repeated stripping. However, dietary lipid and ingredient energy ADCs were significantly affected by repeated stripping. Additionally, the repeated stripping significantly ($P<0.05$) affected the dietary and ingredient AACs for minerals, suggesting that although the collection of feces by repeated manual stripping may be a useful method for the determination of protein ADCs and amino acid AACs, for lipid, energy ADCs and mineral AACs single stripping is a more accurate alternative.
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Keywords: Rainbow trout; Methods; Digestibility; Poultry meal; Stress; Cortisol; *TNF-α* expression

1. Introduction

As fish are cultured in water, problems associated with nutrient leaching from feces into the water column, which lead to an over estimation of nutrient digestibility, have to be overcome for the reliable determination of nutrient digestibility (Windell et al., 1978; Allan et al., 1999). There have been numerous studies investigating the validity of fecal collection methods such as manual stripping, rectal suction, dissection and several settlement techniques, for the determination of nutrient digestibility in fish (Nose, 1960; Cho et al., 1974; Austreng, 1978; Windell et al., 1978; Vens-Cappell, 1985; Weatherup and

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McCracken, 1998; Allan et al., 1999; Storebakken et al., 1999; Ramsay et al., 2000; Percival et al., 2001; Vandenberg and de la Noue, 2001; Hemre et al., 2003). Vens-Cappell (1985) reported nutrient leaching, which was directly related to the time of feces exposure to water when collected from rainbow trout, *Oncorhynchus mykiss*, by filtration from the water column. Logically, this problem is more pronounced when ingredients and feces contain high levels of water-soluble components such as free amino acids, peptides, water-soluble proteins, carbohydrates and minerals.

Two commonly reported methods that are used to avoid the problem of nutrient leaching during fecal collection are dissection of the digestive tract or manual stripping (Austreng, 1978; Weatherup and McCracken, 1998; Allan et al., 1999; Storebakken et al., 1999; Percival et al., 2001; Vandenberg and de la Noue, 2001; Hemre et al., 2003). Compared to dissection, manual stripping has the obvious advantage of being non-lethal to fish. Austreng (1978) compared apparent digestibility values that were determined by either stripping or dissection of fecal matter from rainbow trout, *O. mykiss*, and concluded that there was no difference between methods so long as feces were collected by stripping from the ventral fin to the anus, and by dissection from the posterior half of the rectum. Percival et al. (2001) compared collection of feces by stripping and dissection in Atlantic salmon, *Salmo salar*, and reported significantly lower protein digestibility from fecal samples from dissection, indicating contamination of the feces with indigested food. The manual stripping method appears to be the fecal collection method of choice and has also been compared to the most commonly used method of fecal collection by settlement and, although there have been discrepancies in results; in general results are similar upon comparison (Weatherup and McCracken, 1998; Storebakken et al., 1999; Percival et al., 2001). Unfortunately not all species of fish lend themselves to the manual stripping technique (Allan et al., 1999).

Another common problem faced by researchers conducting digestibility studies is the need to ensure adequate quantities of fecal material are collected for the large array of chemical analyses required to investigate nutrient digestibility and availability. When using manual stripping techniques, adequate material may be obtained by either using a large number of fish, which are sampled once, or a smaller number of fish that are repeatedly sampled until sufficient fecal material is obtained (Austreng, 1978; Weatherup and McCracken, 1998; Storebakken et al., 1999; Percival et al., 2001; Vandenberg and de la Noue, 2001; Hemre et al., 2003; Glencross et al., 2004, 2005; Glencross and Hawkins, 2004). For fecal collection, a typical protocol necessitates that fish are fed for approximately seven days to acclimate to the diets and then feces are collected by manual stripping. If insufficient fecal material is collected, fish may then be fed on the following day with further stripping occurring the day after that. Alternately, fish may be fed for up to a week, until normal feed intake is re-established, before being stripped again. In either case the re-feeding/stripping process is repeated until sufficient fecal material has been collected. Clearly, the handling associated with repetitive sampling of individual fish would be stressful, may result in physical tissue damage (Pickering, 1993; Wendelaar-Bonga, 1997), and as a result, disrupt normal bodily functions, such as immunocompetance (Feveldon et al., 1991; Secombes et al., 2001), and possibly influence digestibility determinations. Unfortunately, there is a lack of published studies investigating the possible effects of repeated fecal collection by manual stripping on fish and digestibility determinations.

A hydrolyzed whole poultry and egg meal product (PEM) was chosen as a model test ingredient for this study as it has the typical attributes of alternative ingredients currently being used to replace fish meal and fish oil. Poultry meal products have been successfully used to replace fish meal in diets for rainbow trout (Alexis et al., 1985; Steffens, 1994). However, the potential for these products to replace fish meal depends on consistency of their digestible nutritional composition, and also on the price of the product. PEM is a by-product of the poultry industry, is produced in significant quantities, and has the potential to replace significant quantities of fish meal and fish oil in diets for rainbow trout.

The aim of this study was to investigate the effects of repeated fecal collection by hand stripping on the digestibility of nutrients from hydrolyzed whole poultry and egg meal by rainbow trout. To determine the physiological impact of multiple strippings we also measured plasma cortisol, the expression of *tumor necrosis factor-α* (*TNF-α*) gene and digestive tract histology to monitor alterations to the stress status, innate immune response, and possible tissue damage brought about by repetitive fecal collection.

2. Materials and methods

2.1. Test ingredient and experimental diets

The analyzed nutrient composition of the experimental batch of hydrolyzed whole poultry and egg meal (PEM) is displayed in Table 2. AgriFeeds Technologies Inc. (Salt Lake City, UT) provided the PEM for testing. PEM is produced in a commercial processes by grinding whole chickens, mixing enzymes and preservatives and then adding eggs, soybean meal as a binder and finally the product is then dried to ~5% moisture. Determination of the digestibility of PEM may indicate its suitability for inclusion into rainbow trout diets.

Table 1
The formulations (g kg^{-1} dry basis) of the reference (REF) and poultry egg meal (PEM) test diets[a]

Item	REF diet	PEM diet
Fish meal[b]	650.0	455.0
Hydrolyzed whole poultry and egg meal (PEM)[c]	–	300.0
Wheat gluten[b]	170.0	119.0
Menhaden fish oil[b]	115.0	80.5
Lignin sulfonate (binder)[d]	15.0	10.5
Vitamin C[e]	10.0	7.0
Vitamin premix[b]	20.0	14.0
Macro mineral premix[b]	12.0	8.4
Trace mineral premix[b]	5.0	3.5
Choline Cl 50%[b]	3.0	2.1
Sum	1000.0	1000.0

[a] Yttrium oxide was added to the diets at a rate of 100 mg kg^{-1}.
[b] Rangen Inc., Buhl, ID, USA.
[c] AgriFeeds Technologies Inc., Salt Lake City, UT, USA.
[d] Permapel plus®, Georga Pacific, Bellingham, WA, USA.
[e] Stay-C® 35, DSM Nutritional Products, Basel, Switzerland.

Table 2
Nutrient composition (dry basis) of the poultry egg meal (PEM) test ingredient and the reference (REF) diet

Item	PEM ingredient	REF diet
Proximate composition		
Dry matter (g kg^{-1})	978.7	927.9
Organic matter[a] (g kg^{-1})	895.2	941.1
Crude protein (g kg^{-1})	563.0	703.8
Crude lipid (g kg^{-1})	157.8	180.3
Energy (MJ kg^{-1})	20.9	23.9
Ash (g kg^{-1})	104.8	58.9
Amino acids (g kg^{-1})		
Alanine	25.8	35.5
Arginine	34.9	46.5
Aspartine	43.9	58.7
Glutamic acid	70.5	129.1
Glycine	35.1	31.1
Histidine	9.4	14.5
Isoleucine	19.3	31.2
Leucine	32.1	53.0
Lysine	23.2	43.6
Methionine	6.0	18.5
Phenylalanine	19.3	29.4
Proline	32.7	41.8
Serine	25.5	25.5
Threonine	18.8	30.2
Tyrosine	14.8	26.8
Valine	23.0	34.5
Minerals (mg kg^{-1})		
Calcium	10,000	11,000
Copper	14	12
Magnesium	2400	1600
Phosphorus	8600	9300
Potassium	19,000	5900
Sodium	8100	4300
Sulfur	13,000	9300

[a] Organic matter (g kg^{-1}) = dry matter (g kg^{-1}) − ash (g kg^{-1}).

The PEM was included into the reference (REF) diet at 30%. The formulation of the REF and 30% PEM diets are displayed in Table 1, and the nutrient composition of the diets are displayed in Table 2. Yttrium oxide was included in both diets (100 mg kg^{-1}) as the inert marker for digestibility determinations. All ingredients were ground or sieved to ensure all particles passed through a 710 μm screen. Dry ingredients were thoroughly mixed in a twin shell V-mixer (Patterson-Kelley, East Stroudsburg, PN, USA) then combined with 330 ml water kg dry mix^{-1} before being cold pelleted through a laboratory pellet mill (California Pellet Mill Co., San Francisco, CA, USA) with a 4-mm die. Pellets were dried at <45 °C in convection drier for approximately 48 h until the moisture content was <10%, to produce a dry sinking pellet.

2.2. Experimental fish

A domesticated strain (Oregon strain, College of Southern Idaho) of rainbow trout, *O. mykiss,* (weight, mean±SE; 308±2.8 g) was used for this study. Rainbow trout were selected from a larger population, counted in groups of 10 fish, weighed, and systematically interspersed into eight 575-l fiberglass tanks to give 50 fish per tank. Four tanks were randomly assigned to each experimental diet. Each tank was supplied with 10-l min^{-1} of untreated, constant temperature (14.5 °C), spring water at the Hagerman Fish Culture Experiment Station, University of Idaho. A fixed photoperiod, controlled by timers and fluorescent lights, was followed (14 h daylight:10 h dark).

2.3. In vivo digestibility procedures

During the course of this study individual fish were exposed to three fecal collection events over a period of five days. The fecal collection procedure is as follows. The fish were acclimated to the experimental conditions for 10 days prior to their first fecal collection event. During this period the fish were fed their respective experimental diet to apparent satiation twice daily (0900 and 1600 h). On the eleventh day, starting at 830 h, fish were captured by net, anaesthetized (MS-222; 80 mg l^{-1} for a maximum duration of 3 min) and feces were collected by manual stripping. The fecal collection procedure took 15 min per tank. Between fecal collection events, fish were returned to their existing tank and fed in the pm of the same day and the am and pm on the following day as described previously and then feces was collected the following morning. Care was taken to avoid contamination of feces with water, mucous and urine. Feces was collected from the fish by first gently wiping the moisture from the bellies of the fish with a paper towel, then using a moderate squeezing motion with the thumb and forefinger, pressure was applied to the abdomen, starting in line at the front of the pelvic fins and finishing at the anus. Feces were collected in a plastic weighing container. To minimize the influence of sampling technique on fecal output between collection series, the same operators were assigned to each set of tanks for each dietary treatment. Fecal material from each day from fish within individual tanks was pooled, weighed (wet and dry), and then analyzed as a single unit. Fish were handled and treated in accordance with the guidelines approved by the Animal Care and Use Committee of the University of Idaho.

2.4. Blood and tissue sampling times

To monitor the stress status and the innate immune response of the fish due to handling and tissue damage brought about by three consecutive fecal collection procedures, blood samples were analyzed for plasma cortisol, liver tissue was analyzed for *TNF-α* gene expression, and stained sections of the distal intestine were microscopically examined for physical damage. Blood, liver and digestive tract tissue samples were only collected from the fish fed the REF diet. The treatment sampling times for blood and tissue collections were as follows:

1) Initial fish (baseline): two fish from each tank were sampled immediately before the first stripping event;
2) Immediately after stripping: two fish were sampled immediately following stripping (15 min);
3) Twenty four hours after stripping: at the completion of the stripping procedure two fish from each tank were transferred into a 575-l tanks and remained unfed and undisturbed and sampled 24 h later;
4) Forty eight hours after stripping: at the completion of the stripping procedure fish were returned to their existing tank and two fish were captured and sampled 48 h later.
5) Fourteen and 21 days after stripping: Liver tissue and digestive tract samples were also collected from two fish from each of the tanks at 14 and 21-day intervals after the first fecal stripping procedure.

2.5. Blood and tissue collection and storage

Blood samples from all fish, except for fish sampled immediately following the stripping procedure, were collected within 1 min of initial disturbance without anesthetic. Blood was collected from the caudal vessels using a 22-gauge 40-mm hypodermic needle and a heparinized 3-ml syringe. Blood was transferred to 2-ml collection tubes and immediately centrifuged (2000 g for 5 min). Plasma was separated and stored at −80 °C until analyzed for cortisol. For the determination of *TNF-α* expression, tissue samples were immediately placed into 2 ml collection tubes containing RNA later (Ambion, Austin, TX, USA). Total RNA was then isolated using TRIzol (Invitrogen, Rockville, MD, USA) according to the manufacturer's protocol, and stored at −80 °C until analyzed. The distal intestinal tissues were fixed in Bouins solution for 48 h and transferred to a 65% ethanol solution until sectioning was carried out. Histological examination was conducted on cross sections and longitudinal sections of tissue taken from distal portions of the intestine. Sections were cut at 5 μm and stained with hematoxylin and eosin. Stained sections of distal intestine were then microscopically (150× magnification) examined for symptoms of physical tissue damage.

2.6. Chemical analyses

Fecal samples were dried in a convection oven at 55 °C for 24 h. Ingredient, diet and fecal samples were analyzed using AOAC (1990) methods for proximate composition, with the exception of crude protein and crude lipid. Dried samples were finely ground by mortar and pestle and analyzed for crude protein (total nitrogen × 6.25) using a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI, USA). Crude fat was analyzed using a soxhlet extraction apparatus (Soxtec System HT, Foss Tecator AB, Hoganas, Sweden) with methylene chloride as the extracting solvent. Ash was determined by incineration at 550 °C in a muffle furnace. Energy content of the samples was determined using a Parr bomb calorimeter (Parr Instrument Co., Moline, IL, USA). Amino acids were analyzed at AAA Laboratories, Mercer Island, WA. The University of Idaho Analytical Sciences Laboratory, Moscow, ID, conducted yttrium and phosphorus analyses, using an Optima 3200 radial inductively coupled plasma atomic emission spectrometer (Perkin-Elmer Corp., Norwalk, CT, USA). Blood samples were analyzed for plasma cortisol using a Correlate-EIA cortisol enzyme immunoassay kit (Assay Designs Inc, Ann Arbor, MI, USA).

To detect the level of *TNF-α* gene expression in liver, real-time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA, USA). The final concentration of each reaction was: Master Mix, 1× (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U μl^{-1}; RNase inhibitor mix, 0.4 U μl^{-1}; forward primer, 300 nM; reverse primer, 300 nM; probe, 200 nM; total RNA, 50 ng. *TNF-α* primers and probe were designed by ABI's Primer Express software using the sequence of trout *TNF-α* (Genbank AJ401377). Cycling conditions for *TNF-α* were as follows: 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, then 40 cycles of PCR consisting of 20 s at 95 °C followed by 1 min at 62 °C. As a cellular mRNA control, *β-actin* levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999). *β-actin* primers and probe were designed using Primer Express software (ABI) using the sequence of trout *β-actin* (Genbank accession number AF254414). Cycling conditions for *β-actin* were the same as those described for *TNF-a*. All samples were run in duplicate. Real-time PCR primer and probe sequences are: *TNF-α* forward, 5'-TGTGTGGCGTTCTCTTAA-TAGCA-3′; *TNF-α* reverse, 5'-CCTGCATCGTTGCCAGTCTT-3'; *TNF-α* probe, 6FAM-CCCTGCTCTTTGCCTGGTGTCAGC-TAMRA; *β-actin* forward, 5'-TGGCCGTACCACCGGTAT-3'; *β-actin* reverse, 5'-GCAGAGCGTAGTCCTCG-TAGATG-3', *β-actin* probe, 6FAM-CTCCGGTGACGGCGTGACCC-TAMRA.

The absolute copy number of each mRNA sample tested was determined by including standards consisting of *in vitro* transcribed mRNAs specific for *TNF-α* or *β-actin* with each set of experimental samples that were analyzed by real-time quantitative RT-PCR. To make standards, primers flanking the real-time PCR fragment were used in RT-PCR of total RNA isolated from liver tissue to amplify a fragment of *TNF-α* or *β-actin* containing the real-time fragment. Primers were designed to contain either a 5' EcoRI site and a 3' BamHI site. Amplified fragments were cloned into pBluescript KS+ (Stratagene, LaJolla, CA, USA) using the EcoRI and BamHI sites. Standards were generated by *in vitro* transcription of the *TNF-α* or *β-actin* clone using Promega's Riboprobe *in vitro* Transcription System (Madison, WI, USA). The transcripts were run on formaldehyde/MOPS gels to confirm the presence of a single band of the correct size, then quantified using a spectrophotometer. The molecular weight of the *in vitro* transcribed RNA was calculated using the following formula: MW = (# of A bases × 328.2) + (# of U bases × 305.2) + (# of C bases × 304.2) + (# of G bases × 344.2) + 159. Using the MW and concentration, copy # μl^{-1} was determined. These transcripts were used as quantitative standards to determine absolute mRNA copy number in each experimental sample. Five serial dilutions of standards were used for each run to generate the standard curve. All quantities were based off of an initial quantification of the isolated *in vitro* transcribed RNA. As such, the absolute numbers given correspond accurately between each run. The primers used to amplify regions for RNA standards synthesis were: *TNF-α* forward INV, 5'-GGATCCAGAGAAAGGCCTCAAGAGGT-3'; *TNF-α* reverse INV, 5'-GAATTCTGCATCGTTGCCAGTCTTCC-3'.

2.7. Apparent digestibility coefficient calculations

Apparent digestibility coefficients (ADC) of diets for dry matter, organic matter, protein and energy, and apparent availability coefficients (AAC) of diets for amino acids and minerals were calculated using the methods described by Maynard and Loosli (1969). Then ADCs and AACs of the PEM were calculated using the methods described by Sugiura et al. (1998).

2.8. Statistical analysis

Homogeneity of variances was assessed using Cochran's *C* test. *TNF-α* gene expression data was $\log_{10}$ transformed to satisfy the assumptions of ANOVA (untransformed data is presented in results). Fecal output, fecal moisture content, dietary ADCs and AACs were analyzed using two-factor ANOVA with diet type (REF and 30% PEM) as the first fixed factor and fecal collection series (1, 2 and 3) as the second fixed factor. For significant two-factor

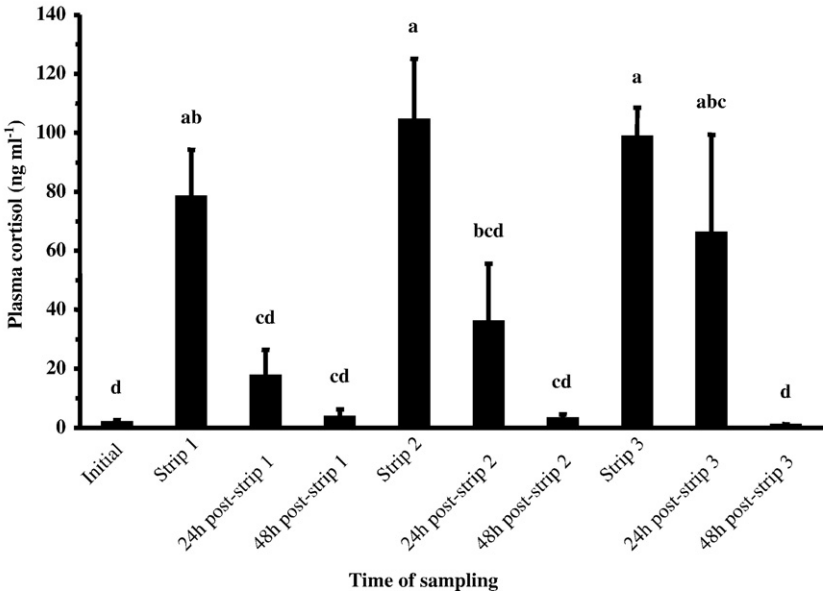

Fig. 1. The plasma cortisol response of rainbow trout fed the reference diet and exposed to repeated fecal stripping procedures. Fish were stripped every 2 days over a five-day period. Means that share the same superscript are not significantly different (mean ± standard error; $n = 8$; $P > 0.05$; one-factor ANOVA; SNK).

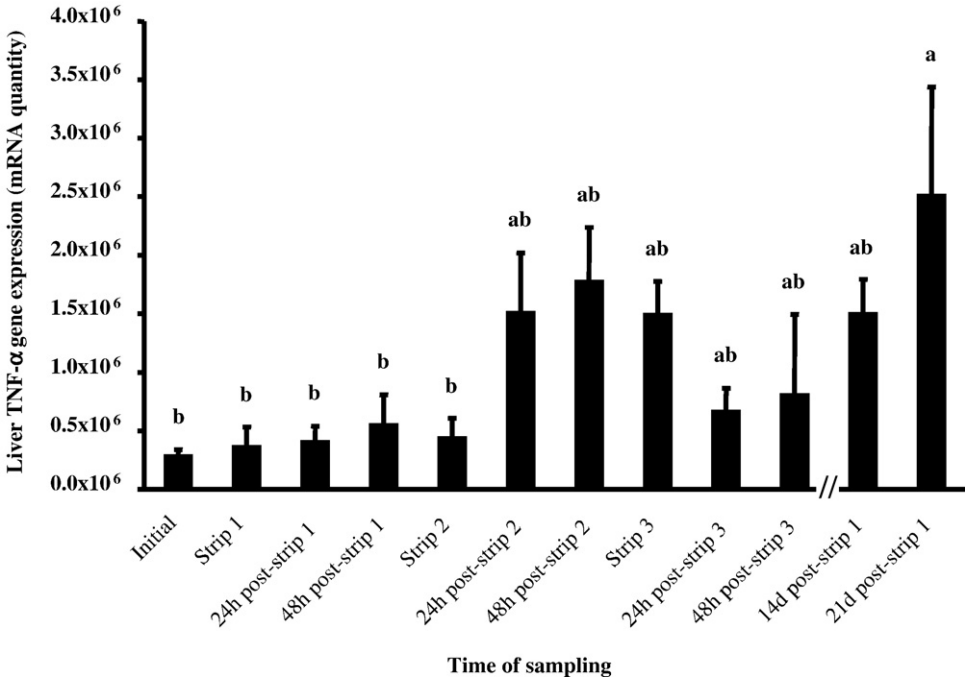

Fig. 2. *Tumor necrosis factor* (*TNF-α*) gene expression by rainbow trout fed the reference diet and exposed to repeated fecal stripping procedures. Fish were stripped every 2 days over a five-day period. Means that share the same superscript are not significantly different (mean±SE; $n=8$; $P>0.05$; one-factor ANOVA; SNK).

ANOVA interactions, data for ingredient ADCs and AACs, plasma cortisol and *TNF-α* expression were compared using one-factor ANOVA. Student Newman–Kuels test was used to identify significant differences between treatment means. All statistical analyses were done using Statistica, Version 6.1 (StatSoft, Inc., Tulsa, OK). Unless otherwise stated all values are reported as the mean±standard error of the mean. A significance level of $P<0.05$ was used.

3. Results

3.1. General observations

Fish were fed approximately 2.6% of their body weight per day during the experiment and readily accepted the diets at every feeding time. There were no visual signs of excessive scale loss, external disease or mortalities during, or up to 21 days following, the experiment. One fish, out of 96 sampled, displayed signs of hemorrhaging of the epithelium of the distal intestine immediately after the third stripping event.

3.2. Plasma cortisol levels

The fecal collection procedures induced an acute cortisol stress response in rainbow trout (Fig. 1). Plasma cortisol levels were significantly elevated at the completion of each stripping event and returned to basal levels within 48 h of each stripping event ($P>0.05$). Although there appeared to be a compounding effect of the fecal collection procedure on plasma cortisol levels 24 h following stripping the response between 24 h post-strip groups was not statistically

Table 3
Results for fecal output, and proximate dietary nutrient ADCs (%) for rainbow trout exposed to repeated fecal collection procedures

Item	First factor: Diet type (A)				Second factor: Fecal collection series (B)					Interaction (A×B)
	REF	PEM	SE	*P*	1	2	3	SE	*P*	*P*
Fecal output										
Wet (g kg fish^{-1})	3.60	4.48	0.13	<0.001	3.79^{b}	3.94^{ab}	4.40^{a}	0.16	0.036	0.152
Dry (g kg fish^{-1})	0.37	0.38	0.01	0.559	0.34^{b}	0.38^{b}	0.42^{a}	0.01	0.003	0.027
Dietary ADC (%)										
Dry matter	84.43	77.78	0.31	<0.001	81.15	80.71	81.45	0.38	0.410	0.327
Organic matter	87.56	81.79	0.12	<0.001	84.53	84.78	84.72	0.15	0.464	0.734
Crude protein	93.43	90.82	0.08	<0.001	92.08	92.19	92.10	0.09	0.666	0.053
Crude lipid	95.14	91.30	0.37	<0.001	91.97^{b}	94.48^{a}	93.19^{ab}	0.45	0.004	0.035
Energy	87.01	81.55	0.21	<0.001	84.78	84.59	84.51	0.25	0.730	0.085

Two-factor ANOVA results: For the first factor (A), diet type (reference diet (REF) or poultry egg meal diet (PEM)) ($P<0.05$; mean±pooled standard error; $n=12$; two-factor ANOVA). For the second factor (B), fecal collection series (series 1, 2 or 3), values within the same row that share the same superscript are not significantly different ($P>0.05$; mean±pooled standard error; $n=8$; two-factor ANOVA; SNK).
Significant interactions between diet type and fecal collection series (A×B) for dry fecal output and the ADCs of dietary lipid are explained in Table 4.

Table 4
The explanation of the two-factor ANOVA interactions for dry fecal output and dietary lipid ADCs (%), and the poultry egg meal (PEM) ingredient ADCs (%) by rainbow trout exposed to repeated fecal collection procedures

Item	Fecal collection series			SE	*P*
	1	2	3		
	ADC %	ADC %	ADC %		
Interaction of dry fecal output					
Diet 1 Ref. (g kg fish^{-1})	0.30^{b}	0.39^{a}	0.43^{a}	0.02	<0.001
Diet 2. PEM (g kg fish^{-1})	0.38	0.36	0.41	0.02	0.370
Interaction of dietary ADC for lipid					
Diet 1. Ref.	93.15^{b}	96.17^{a}	96.11^{a}	0.25	<0.001
Diet 2. PEM	90.85	92.78	90.27	0.87	0.155
PEM ingredient ADC (%)					
Dry matter	64.69	62.80	62.61	1.56	0.609
Organic matter	68.04	67.60	67.29	0.87	0.833
Crude protein	84.55	82.75	82.38	0.66	0.093
Crude lipid	84.72	83.74	74.69	3.18	0.098
Energy	69.17^{a}	60.31^{b}	65.86^{a}	1.35	0.004

Values within the same row sharing the same superscript are not significantly different (mean±pooled standard error; $n=4$; $P>0.05$; one-factor ANOVA; SNK).

significant. There was however, significant evidence of heterogeneity of variances for the plasma cortisol response of 24 h post-strip groups, with variances increasing with successive collections ($P<0.05$).

3.3. TNF-α expression

The expression of *TNF*-α indicated a compounding chronic effect of the repeated stripping procedure on the innate immune response of the fish (Fig. 2). Compared to initial *TNF*-α expression, there was evidence of an elevation in *TNF*-α expression 24 h following the second stripping event. *TNF*-α expression remained significantly elevated 21 d after the first stripping event ($P<0.05$).

3.4. Distal digestive tract histology

Histological examination of intestines of fish revealed no apparent evidence of physical damage to the distal digestive tract due to striping at any time throughout the experiment. There were no signs of degeneration or trauma to the villi in fish of any treatment. While some lesions were seen in the muscularis, both the circular and longitudinal layers, they are thought to be due to the trauma of dissection and not stripping since they were seen in approximate equal numbers of tissues from controls and test fish.

3.5. Fecal output and moisture content

There were significant effects of diet type on (REF diet<PEM diet) and fecal collection series (Collection 3>2>1) on wet fecal output ($P<0.05$; Table 3). There was no significant interaction between diet type and fecal collection series for wet fecal output. There was no significant effect of diet type on dry fecal output (Table 3). However, there was a significant effect of fecal collection series on dry fecal output. There was also a significant interaction between diet type and fecal collection series for dry fecal output. The interaction may be explained by the significant ($P<0.05$; Table 4) increase in dry fecal output from fish fed the REF diet (series 1<series 2=series 3), while the dry fecal output of fish fed the PEM diet remained constant ($P>0.05$).

3.6. Dietary digestibility and availability of nutrients

There was a significant effect of diet type on dietary ADCs for dry matter, organic matter, protein and energy (Table 3). Dietary ADCs for

Table 5
Results for dietary amino acid AACs (%) and for PEM ingredient amino acid AACs (%) for rainbow trout exposed to repeated fecal collection procedures

Item	Two-factor ANOVA diet[1]						One-factor ANOVA ingredient AACs[2]				
	Diet type AAC (%)						Fecal collection series AAC (%)				
	REF	PEM	SE	*P* (A)	*P* (B)	*P* (A×B)	1	2	3	SE	*P*
Alanine	95.21	92.84	0.30	<0.001	0.887	0.308	86.98	81.87	86.80	2.96	*0.418*
Arginine	98.09	96.91	0.10	<0.001	0.165	0.788	92.60	93.70	93.48	0.59	*0.422*
Aspartine	94.01	91.60	0.21	<0.001	0.900	0.074	86.90	84.88	80.48	1.63	*0.550*
Glutamic acid	97.87	95.66	0.07	<0.001	0.238	0.066	88.34^{a}	86.08^{ab}	84.20^{b}	0.72	*0.009*
Glycine	93.90	91.83	0.22	<0.001	0.543	0.930	87.74	87.49	87.45	1.00	*0.976*
Histidine	96.42	95.79	0.14	<0.001	0.561	0.422	93.81	94.81	91.99	1.34	*0.360*
Isoleucine	96.76	95.53	0.14	<0.001	0.202	0.947	90.54	90.78	91.33	1.12	*0.878*
Leucine	97.43	95.89	0.13	<0.001	0.193	0.871	89.11	90.46	90.21	1.08	*0.656*
Lysine	96.62	96.39	0.17	0.340	0.537	0.823	95.21	94.68	96.24	1.86	*0.836*
Methionine	97.58	97.59	0.12	0.957	0.500	0.684	98.93	97.62	96.41	1.32	*0.435*
Phenylalanine	95.20	93.80	0.34	0.010	0.966	0.166	91.58	91.64	88.31	1.52	*0.327*
Proline	96.83	93.88	0.21	<0.001	0.276	0.476	86.58	84.36	84.39	1.24	*0.386*
Serine	96.65	93.04	0.19	<0.001	0.537	0.377	83.91	82.65	81.12	0.96	*0.175*
Threonine	95.92	93.98	0.21	<0.001	0.357	0.440	88.49	86.60	84.95	1.46	*0.279*
Tyrosine	97.13	96.23	0.18	0.003	0.220	0.888	92.95	92.27	91.98	1.86	*0.931*
Valine	96.05	93.65	0.13	<0.001	0.141	0.765	85.46	84.65	85.64	1.09	*0.796*

[1]Two-factor ANOVA results: For the first factor (A), diet type (reference diet (REF) or poultry egg meal diet (PEM)) ($P<0.05$; mean±pooled standard error; $n=12$; two-factor ANOVA). For the second factor (B), fecal collection series (series 1, 2 or 3), there were no significant effects of fecal collection series ($P>0.05$; mean± pooled standard error; $n=8$; two-factor ANOVA). There were no significant interactions (A×B) between diet type and fecal collection series ($P>0.05$).
[2]One-factor ANOVA results: Means within the same row sharing the same superscript are not significantly different ($P>0.05$; mean±pooled standard error; $n=4$; one-factor ANOVA; SNK).

Table 6
Results for dietary minerals AACs (%) for rainbow trout exposed to repeated fecal collection procedures[1]

AAC (%)	First factor: Diet type (A)				Second factor: Fecal collection series (B)					Interaction (A × B)[2]
	REF	PEM	SE	*P*	1	2	3	SE	*P*	*P*
Ash	28.09	33.99	0.53	<0.001	33.30[a]	29.50[b]	30.33[b]	0.65	0.002	0.027
Calcium	2.19	6.17	1.41	0.056	11.69[a]	0.70[b]	0.15[b]	1.64	<0.001	0.015
Copper	39.76	50.00	0.92	<0.001	44.96	45.56	44.13	1.12	0.671	0.002
Magnesium	51.15	60.39	1.38	<0.001	67.03[a]	48.44[b]	51.84[b]	1.69	<0.001	0.128
Phosphorus	51.72	61.11	0.74	<0.001	62.20[a]	54.20[b]	52.85[b]	0.91	<0.001	0.009
Potassium	94.61	94.39	0.10	0.142	94.38	94.53	94.61	0.13	0.445	0.001
Sodium	−22.40	−72.23	3.94	<0.001	−44.95	−51.95	−45.04	4.82	0.153	0.142
Sulfur	72.86	70.70	0.67	0.040	73.89[a]	71.01[b]	70.52[b]	0.82	0.021	0.051

[1]For the first factor (A), diet type (reference diet (REF) or poultry egg meal diet (PEM)) ($P<0.05$; mean±pooled standard error; $n=12$; two-factor ANOVA). For the second factor (B), fecal collection series (series 1, 2 or 3), values within the same row that share the same superscript are not significantly different ($P>0.05$; mean± pooled standard error; $n=8$; two-factor ANOVA; SNK).
[2]Significant interactions (A × B) between diet type and fecal collection series are explained in Table 6.

dry matter, organic matter, protein and energy were significantly higher for the control diet than the PEM diet. There was no significant effect of fecal collection series on dietary ADCs for dry matter, organic matter, protein or energy. There were also no significant interactions between diet type and fecal collection series for these indices.

For dietary lipid ADCs, there was a significant effect of diet type (Ref>PEM; $P<0.05$; Table 3) and fecal collection series (series 1=series 3≥series 2), and there was also a significant interaction between the two factors. The interaction may be explained by the slight, but significant ($P<0.05$; Table 4), increase in dietary lipid ADCs (Table 4; series 1<series 2=series 3) for fish fed the REF diet, while the dietary lipid ADCs from fish fed the PEM diet remained constant between fecal collections ($P>0.05$).

With the exception of lysine and methionine, there was a significant effect ($P<0.05$; Table 5) of diet type on dietary AACs (REF>PEM diet). There were no significant effects of fecal collection series or significant interactions observed for the AACs of any dietary amino acids.

With the exceptions of calcium and potassium, there was a significant effect of diet type on dietary mineral AACs (Table 6; $P<0.05$). Dietary AACs for sulfur and sodium were significantly higher in the REF diet than the PEM diet. Ash, copper, magnesium and phosphorus AACs were significantly higher in the PEM diet. For fecal collection series, there was a significant negative effect on the dietary AACs of ash, calcium, magnesium, phosphorus and sulfur due to repeated stripping. There were significant interactions between diet type and fecal collection series for ash, calcium, copper, phosphorus, potassium and sulfur dietary AACs.

The interactions for ash and calcium may be explained by the significant reduction in dietary AACs for the REF diet, while the dietary AACs for these minerals remained constant in the PEM diet (Table 7). The phosphorus interaction may be explained by the more pronounced reduction in dietary AACs of the REF diet series compared to the PEM diet series. The interaction for the dietary copper AACs is due to an increase in AACs for the REF diet series as opposed to corresponding decrease for the PEM diet series due to repeated stripping. For potassium, dietary ACCs for fish fed the REF diet exhibited a significant increase while the AACs for the PEM fed fish remained constant throughout the repeated stripping procedures.

3.7. PEM ingredient nutrient digestibility and availability

There were no significant effects of fecal collection series on ingredient ADCs for dry matter, organic matter, protein or lipid ($P>0.05$; Table 4). However, there was a significant effect of fecal collection series on ingredient ADCs for energy ($P<0.05$; series 1=series 3>series 2).

There were no significant effects of repeated stripping on the ingredient AACs of any amino acids from the PEM ($P>0.05$; Table 5).

Ingredient AACs for copper, phosphorus, potassium, sodium, and sulfur were significantly affected by repeated stripping ($P<0.05$; Table 7). AACs for copper, potassium, sodium, and sulfur were significantly reduced due to repeated stripping, while phosphorus ingredient AACs were significantly higher at the second stripping. There were no significant effects of repeated stripping on ingredient AACs for ash, calcium or magnesium ($P>0.05$).

Table 7
Explanations of the interaction between diet type and fecal collection series for dietary mineral availability from Table 4, and the PEM ingredient AACs (%) for minerals by rainbow trout exposed to repeated fecal stripping procedures

Item	Fecal collection series 1 AAC (%)	Fecal collection series 2 AAC (%)	Fecal collection series 3 AAC (%)	SE	*P*
Dietary mineral interactions					
Ash					
Diet 1: REF	31.91[a]	26.02[b]	26.35[b]	0.79	<0.001
Diet 2: PEM	34.69	32.98	34.31	1.03	0.498
Calcium					
Diet 1: REF	13.79[a]	−5.11[b]	−2.11[b]	2.34	<0.001
Diet 2: PEM	9.60	6.51	2.41	2.28	0.173
Copper					
Diet 1: REF	36.94[b]	39.52[ab]	42.84[a]	1.12	0.015
Diet 2: PEM	59.97[a]	51.59[ab]	45.42[b]	1.95	0.497
Phosphorus					
Diet 1: REF	55.70[a]	41.99[b]	42.99[b]	1.54	<0.001
Diet 2: PEM	64.11[a]	61.14[ab]	57.53[b]	1.17	0.006
Potassium					
Diet 1: REF	94.13[b]	94.59[ab]	95.13[a]	0.18	0.011
Diet 2: PEM	94.63	94.47	94.08	0.17	0.120
PEM ingredient: mineral AAC (%)					
Ash	38.22	41.83	44.42	2.34	0.225
Calcium	11.04	36.34	14.01	10.40	0.225
Copper	91.49[a]	78.50[b]	50.36[c]	3.57	<0.001
Magnesium	70.13	71.87	69.99	3.14	0.916
Phosphorus	77.14[b]	96.14[a]	81.11[b]	4.11	0.023
Potassium	95.01[a]	94.37[a]	93.31[b]	0.29	0.009
Sodium	−89.84[a]	−120.88[b]	−159.04[c]	6.13	<0.001
Sulfur	73.95[a]	69.81[a]	57.91[b]	3.41	0.022

Means within the same row with the same superscript are not significantly different (mean±pooled standard error; $n=4$; $P>0.05$; one-factor ANOVA; SNK).

4. Discussion

The goal of this study was to determine if repeated sampling of rainbow trout induces stress, alters the innate immune system, causes physical damage to the distal section of the digestive tract, and ultimately affects nutrient digestibility and availability determinations. Plasma cortisol has been used as a reliable indicator of acute stress in fish (Pickering, 1993; Wendelaar-Bonga, 1997). Plasma cortisol levels of fish were elevated at the completion of each fecal collection period, indicating the fish were stressed by repetitive sampling procedure. There did appear to be a compounding effect of the repeated fecal collection procedure on the cortisol stress response of fish throughout our study. This suggests that some individual fish did not adapt to the repeated sampling protocol, and may also indicate the manifestation of tissue damage in some fish from handling. There were no histological alterations to the tissue of the distal section of the digestive tract evident for any fish, indicating that the fecal collection procedure did not damage the epithelial cells of the digestive tract. There was however, evidence of elevated TNF-α expression. TNF-α is a pro-inflammatory cytokine whose expression is elevated following injury or infection. Expression of TNF-α has been used as a reliable indicator for up-regulation of the innate immune response (Secombes et al., 2001) and for tissue damage (Cho et al., 2001; Willuweit et al., 2001). The elevated expression of TNF-α found in this study provides evidence of cellular damage occurring in rainbow trout due to repeated stripping. There appeared to be a compounding effect of successive stripping on *TNF-α* gene expression in the liver, such that significant higher levels were found in animals 21 days after stripping. From samples obtained in this study it is not possible to ascertain the exact location of tissue damage occurring in these fish, specifically whether it is occurring in cells lining the peritoneal cavity due to stripping or possibly the intestinal cells of the fish. As there were no visual symptoms of disease or mortalities that occurred during the handling of the fish or up to 21 days following the treatment, it is likely that the elevated level of TNF-α detected in the liver of these fish is due to undiagnosed internal tissue damage. Our results found that TNF-α expression levels appeared to increase between the first and second post-stripping events, however the variability between the samples analyzed was too large to determine a significant difference.

There was a trend, although not statistically significant, of a reduction of fecal moisture content with successive stripping in our study. This suggests that repeated stripping may have influenced the rate of passage of the chyme through the digestive tract, which in turn may influence digestibility. Percival et al. (2001) noted reduced moisture content of feces and numerically lower ADCs for organic matter and crude protein by Atlantic salmon, *S. salar*, which were stripped twice during the same fecal collection period.

The digestibility of protein and availability of amino acids from diets and ingredients remained relatively unaffected by repeated sampling of feces. This is not particularly surprising given that for salmonids, the majority of the digestion of protein, absorption of low molecular weight peptides, and amino acids takes place in the pyloric region of the digestive tract (Fänge and Grove, 1979; Buddington and Diamond, 1987; Buddington and Hilton, 1987; Kuz'mina, 1996; Krogdahl et al., 1999; Nordrum et al., 2000). Therefore, protein digestion and amino absorption would have been relatively complete by the time the chyme reached the primary target of manual stripping, the distal section of the intestine.

Results were not as consistent for ingredient energy ADCs or dietary and ingredient lipid ADCs. Dietary lipid ADCs increased with successive stripping events, while there was a numerical trend for the reduction of ingredient lipid digestibility with each successive stripping event. Additionally, changes in lipid ADCs did not appear to relate directly to energy ADCs. The inconsistencies in lipid and energy ADCs due to repeated stripping are difficult to explain, but may be associated with several factors, either alone or in combination, such as handling stressors inducing disturbances in feed intake or gut passage rate of chyme or dietary lipid and carbohydrate interactions. Bergot and Breque (1983) reported a reduction in ADCs for dry matter, starch and protein from rainbow trout as feed intake levels increased from 0.5% to 1% fish live weight per day. Storebakken et al. (1998) reported significant interactions between carbohydrate source and concentration for ADCs of lipid by rainbow trout. Diets with 15% carbohydrate resulted in higher ADCs of lipid than diets with 25% carbohydrate when lipid levels were held constant at 18% (Storebakken et al., 1998). We did not measure dietary carbohydrate content, carbohydrate digestibility or feed intake so we cannot draw direct comparisons. We did, however, observe excellent feeding activity by the fish regardless of previous handling and stripping events, which may suggest that feed intake was not a factor. Further work is needed to delineate the findings.

With the exception of calcium and magnesium AACs, dietary mineral availabilities were significantly altered with each subsequent fecal collection. The distal section of the digestive tract is known to be a site of osmoregulation and mineral absorption and excretion in fish (Smith, 1980). Stress has been demonstrated to cause osmoregulatory dysfunction in fish (Pickering, 1993; Wendelaar-Bonga, 1997). It is possible that a combination of stress, and possible internal physical damage caused by the repeated fecal collection procedure resulted in osmoregulatory imbalances, as evident by shifts in fecal moisture content, that impacted mineral metabolism and ultimately AAC values.

Digestibility data obtained from this study indicates that PEM is lower in digestible protein (48%) and higher in digestible lipid (13%) when compared to digestible values obtained using the settlement technique for herring meal (64 and 8%), menhaden meal (53 and 8%), feed grade poultry meal (52 and 9%), prime poultry meal (56 and 7%) and refined poultry meal (61 and 8%) reported for rainbow trout by Cheng and Hardy (2002). Additionally, all digestible essential amino acid levels in PEM were lower when compared to herring meal and menhaden meal and poultry meals reported for rainbow trout by Cheng and Hardy (2002). While the digestible energy level of PEM (14 MJ kg^{-1}) was comparable to menhaden meal (13 MJ kg^{-1}), it was lower than herring meal (19 MJ kg^{-1}), feed grade poultry meal (17 MJ

kg^{-1}), prime poultry meal (16 MJ kg^{-1}) and refined poultry meal (17 MJ kg^{-1}) (Cheng and Hardy, 2002). While drawing these comparisons it is important to consider that ADCs determined via settlement are systematically overestimated, whereas, ADCs determined via stripping are systematically underestimated.

On face value, the gross nutritional composition of PEM appears favorable when compared to fish meal and other poultry by-product meals (NRC, 1993; Sugiura et al., 1998; Cheng and Hardy, 2002). In spite of this, ADC and AAC results indicate that PEM is of slightly lower nutritional value to rainbow trout than herring meal, menhaden meal, standard feed grade poultry meal and more refined poultry products reported by Cheng and Hardy (2002). However, PEM does have the advantage of containing lower levels of unavailable phosphorus (0.2%) for rainbow trout than the aforementioned meals (Cheng and Hardy, 2002).

5. Conclusion

The repeated fecal collection procedure used in this study induced a cortisol stress response and also resulted in an elevation of *TNF-α* expression, indicating a pro-inflammatory cytokine response in rainbow trout. However, both macroscopic and microscopic examinations revealed no pathology, minor scale loss, minimal external skin damage and no evidence of histological alteration to the distal section of the digestive tract. Ultimately the ADCs for protein and AACs for amino acids remained unaffected by repeated fecal collection procedures throughout the study, while apparent lipid and energy ADCs and mineral AACs were impacted. This leads us to conclude that for rainbow trout, the collection of feces by repeated manual stripping may be a useful method for the determination of protein ADCs and amino acid AACs. However, due to the significant effects of repeated stripping on lipid and energy ADCs and mineral AACs, we recommend a fecal collection routine that uses a longer time between repeated stripping events or single stripping as a more accurate alternative for these indices.

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